YU-PANG LIN. Identification and quantitation of DNA adducts from calf thymus DNA exposed to 1,2-epoxybutene (under the direction of James A. Swenberg)

Abstract

This study was conducted to characterize the DNA adducts formed when adenine, guanosine or calf thymus DNA is reacted with 1,2-epoxybutene (EB). HPLC separation coupled with diode-array detection was employed to isolate the adducts that were formed. Two EB-adenine products were identified as N^6-(2-hydroxy-3-buten-1-yl) adenine (EB-Ade I) and N^6-(1-hydroxy-3-buten-2-yl) adenine (EB-Ade II) by ^{1}H-NMR analysis. Two guanine adducts similar to those previously characterized as 7-(2-hydroxy-3-buten-1-yl) guanine (EB-Gua I) and 7-(1-hydroxy-3-buten-2-yl) guanine (EB-Gua II) were also collected. All four adducts were used as reference compounds to detect and quantitate the corresponding adduct species formed in DNA incubated *in vitro* with EB. EB-adenine and EB-guanine adducts were detected in EB-treated calf thymus DNA. The formation of EB-adenine adducts (1.0×10^{-3} and 2.7×10^{-3} EB-Ade I and II per adenine, respectively) in calf thymus DNA was approximately one tenth that of EB-guanine adducts (4.2×10^{-2} and 3.0×10^{-2} EB-Gua I and II per guanine, respectively). Since 1,3-butadiene (BD) and its metabolites, EB and diepoxybutane, induce nearly equal numbers of point mutations at A:T and G:C base pairs, the N^6-substituted adenine adducts may represent important promutagenic DNA adducts involved in BD-induced mutagenesis and carcinogenesis.

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LIST OF ABBREVIATIONS

BD	1,3-butadiene
CYP2E1	cytochrome P450 2E1 monooxygenase
DAD	diode-array detector
DEB	diepoxybutane
EB	1,2-epoxybutene
EBD	3,4-epoxy-1,2-butanediol
EB-Ade I	N^6-(2-hydroxy-3-buten-1-yl) adenine
EB-Ade II	N^6-(1-hydroxy-3-buten-2-yl) adenine
EB-Gua I	7-(2-hydroxy-3-buten-1-yl) guanine
EB-Gua II	7-(1-hydroxy-3-buten-2-yl) guanine
FR	flow rate
HPLC	high performance liquid chromatography
LSIMS	liquid secondary ionization mass spectra
MS/MS	tandem spectrometry
NMR	nuclear magnetic resonance
NTH	neutral thermal hydrolysate
SPE	solid phase extraction
TLC	thin layer chromatography
UV	ultraviolet

INTRODUCTION

1,3-Butadiene (BD), a colorless gas, has been mainly used in the manufacture of synthetic rubber. Emission of BD may occur during its manufacture, storage, transport, use or disposal. BD has also been found in automobile exhaust [1] and cigarette smoke [2]. BD is listed as one of 189 hazardous air pollutants in the 1990 Clean Air Act Amendments [3].

The International Agency for Research on Cancer has classified BD as a group 2A carcinogen [4] with sufficient evidence for carcinogenicity in experimental animals, but limited evidence for carcinogenicity of BD in humans. Epidemiological studies of workers exposed to BD provide controversial evidence with the results showing the association of BD exposure with lymphatic and hematopoietic cancers despite an overall deficit in cancer mortality [5,6]. Recent findings in rodent inhalation carcinogenicity studies of BD have demonstrated the strikingly high sensitivity of mice to BD-induced tumors compared to rats [7-9]. Low exposure of BD, even at 6.25 ppm, increased incidences of tumors in female B6C3F1 mice; in contrast, increased incidences of tumors were only seen in Sprague-Dawley rats exposed to 1000 to 8000 ppm BD. The marked species difference in carcinogenic potency of BD is nearly three orders of magnitude higher in mice than rats, and hence BD is a potent carcinogen in mice and a weak carcinogen in the rat.

According to several studies on the metabolism of BD, BD is first metabolized by cytochrome P450 2E1 monooxygenase (CYP2E1) to 1,2-epoxybutene (EB), and then EB can be either oxidized further to diepoxybutane (DEB) or hydrolyzed via epoxide hydrolase to 3-butene-1,2-diol, which can be subsequently metabolized to 3,4-epoxy-1,2-butanediol (EBD) via cytochrome

P450 [10-13]. The elimination of BD may either proceed by glutathione conjugation to EB or DEB, or by hydrolysis of EB or DEB [13].

BD mutagenicity in the presence of metabolic activation has been demonstrated in the *S. typhimurium* assay. The reactive metabolites EB and DEB are strongly mutagenic in test systems such as Salmonella, Saccharomyces, and Drosophila [14,15]. The mutagenicity of all three metabolites, EB, EBD and DEB, has been shown at both *tk* and *hprt* loci in cultured TK6 human lymphoblastoid cells [16]. Similarly, BD , EB and DEB were mutagenic in splenic T cells from exposed B6C3F1 mice [17]. Increases in sister chromatid exchange, chromosomal aberrations, and micronucleated peripheral blood erythrocytes were found after *in vivo* exposure to BD [18,19]. Exposure of BD also caused covalent binding to hemoglobin [20] and nuclear proteins [21,22] and protein-DNA and DNA-DNA crosslinks [23] in mice.

As the major metabolite of BD metabolism, EB has been shown to react with deoxyguanosine and *in vitro* DNA to form two main adducts identified as 7-(2-hydroxy-3-buten-1-yl)guanine (EB-Gua I) and 7-(1-hydroxy-3-buten-2-yl)guanine (EB-Gua II) [24]. DEB has also been shown to react with guanine to give an adduct, 7-N-(2,3,4-trihydroxybutyl)guanine [23]. EB-Gua II and 7-N-(2,3,4-trihydroxybutyl) guanine were detected, but not quantitated in the liver DNA hydrolysate from B6C3F1 mice exposed to 500 ppm (1,4-^{14}C)1,3-butadiene; however, they were not detected in DNA from rats after exposure to ^{14}C-BD [23].

The results of the mutational spectrum induced by EB in splenic T-cells of male B6C3F1 mice have shown four A:T→G:C transitions and one A:T→C:G transversion among 18 basepair substitutions [17]. Furthermore, nearly half of the point mutations induced by BD, EB, and DEB occured at A:T base pairs [17]. Additionally, two studies on the frequency of mutations in the bone marrow of B6C3F1 *lacI* transgenic mice have demonstrated increased point mutations at A:T

base pairs following BD inhalation exposures [25,26]. These in vivo data suggest that adenine DNA adducts may be involved in BD mutagenesis and carcinogenesis. The purpose of the present study is to characterize two main adducts that are formed from the reaction of EB with adenine, and to use synthesized EB-guanine and EB-adenine adducts as reference compounds to detect and quantitate the corresponding adduct species formed in DNA incubated *in vitro* with EB.

MATERIALS AND METHODS

Chemicals

EB (98%) was obtained from Aldrich (Milwaukee, WI). Glacial acetic acid was bought from EM Science (Gibbstown, NJ). Adenine, guanine, guanosine, cacodylic acid and calf thymus DNA were purchased from Sigma (St. Louis, MO). Acetone, diethyl ether, methanol, and HCl were purchased from Mallinckrodt (Paris, KY). Acetic acid, ammonium formate, Tris, potassium dihydrogen phosphate, and potassium hydroxide were bought from Fisher Scientific (Pittsburg, PA). C18 reverse phase resin and phosphorus pentaoxide were purchased from Baker (Phillipsburg, NJ).

High Performance Liquid Chromatography (HPLC)

A C18 reverse phase column (described below in each section of methods), a Rhodyne injector (Baxter, Charlotte, NC) equipped with a 2-ml injection loop, 2 Waters 510 HPLC pumps (Millipore, Milford, MA), and one of two detection systems were used for the analysis of some reaction products: (1) a Hewlett Packard 1040A diode-array detector (DAD) equipped with a HPLC-detection computer system, (2) an Applied Biosystems, Inc. (Ramsey, NJ) 757 absorbance detector. The eluents used in the HPLC separation of the reaction products in a reaction mixture are described below.

Mass Spectrometry

Liquid secondary ionization mass spectrometry (LSIMS) was conducted to obtain mass spectra of analytes using a VG 70-250SEQ hybrid mass spectrometer equipped with a Cs^+ gun operating at a primary ion beam of 30 kV. The Cs^+ ions were allowed to bombard the sample dissolved in a matrix containing a mixture

of methanol and water. Tandem spectrometry (MS/MS) studies were performed by mass-selecting positive ions from the magnetic analyzer and allowing them to collide with neutral Ar atoms (pressure 2 x 10^{-5} torr) present in the first quadrupole at a collision energy of 50 eV followed by the mass analysis with the second quadrupole.

Reaction of EB with Guanosine

The procedure used was modified from that of Citti *et al.* [24]. Guanosine (105 mg, 0.37 mmol) was reacted with EB (266 mg, 3.72 mmol) in 1.75 ml acetic acid (diluted from equal volume, 0.875 ml, of distilled water). The reaction mixture was heated at 50°C for 5 hours. The reaction was stopped with 1.75 ml acetone and 7.0 ml diethyl ether. The precipitate after centrifugation in a Centra-8R Tabletop centrifuge (International Equipment Company, Needham Heights, MA) at 400 rpm (20x *g*) for 5 minutes was dissolved in 20 mM potassium phosphate pH 5.5 (2 ml). This product solution was hydrolyzed by heating at 80°C for 5 hours in 1 N HCl, and neutralized with 1 N KOH and then frozen until later HPLC analysis. The HPLC column used in the separation of EB-guanosine hydrolysate was a Baxter (McGaw Park, IL) B&J OD5 octadecyl analytic column (25 x 0.46 cm, 5mm). The eluents used in HPLC were (a) 70% aqueous methanol (degassed) and (b) 20 mM potassium phosphate buffer, pH 5.5 (degassed). The conditions of flow rate (FR) and gradient in the HPLC separation were: (1) FR 1.5 ml/min, and (2) a linear gradient from 15% (a) and 85% (b) to 50% of each over 30 minutes. Purified fractions at the same retention time were pooled into a solution and a small portion of the pooled solution was used for HPLC analysis with DAD to obtain a purity index (spectra match value which ranges from 0 to 1000, indicating no change in absorption spectra at consecutive time points of a peak) for the pooled fractions. The fractions of

interest were freed from phosphate salts by applying to the HPLC column and washing with degassed distilled water for the first 30 min and then a linear gradient from 100% degassed distilled water to 100% degassed methanol in 5 min. The fractions of interest were collected and then evaporated *in vacuo* to drynesss by a Savant (Farmingdale, NY) svc100 speed-vac and then dissolved in distilled water to measure λ_{max} and λ_{min} with a Shimadzu (Columbia, MD) UV-160U spectrophotometer. After drying again through the speed-vac, the fractions were subjected to LSIMS and MS/MS.

Reaction of EB with Adenine

Adenine (50 mg, 0.37 mmol) was reacted with EB (266 mg, 3.72 mmol) in a mixture of 15 ml 10 mM Tris-HCl pH 7.2 with 15 ml methanol. The reaction mixture was heated at 50°C for 5 hours. Unreacted EB was extracted from the reaction mixture by adding and decanting 120 ml diethyl ether twice. The remaining reaction mixture was frozen until later HPLC analysis. Primary separation of EB-adenine products was conducted with a Beckman (Fullerton, CA) octadecyl semi-preparative column (25 x 1 cm, 5 mm) coupled with DAD. The eluents used in primary HPLC separation were (a) 70% aqueous methanol (degassed) and (b) 20 mM potassium phosphate buffer, pH 5.5 (degassed). The other conditions of the primary HPLC separation were: (1) FR 3.0 ml/min, and (2) a linear gradient from 15% (a) and 85% (b) to 50% of each over 30 minutes. Purified fractions from the same retention time were pooled and a small portion of the pooled solution was used for the secondary HPLC analysis with a Beckman octadecyl analytic column (25 x 0.46 cm, 5 mm) and DAD to obtain a purity index for the pooled fractions. The secondary HPLC analysis was conducted under the same conditions as described in the previous primary separation except a change of FR to 1.5 ml/min. The fractions of interest were

freed from phosphate salts by washing in solid phase extraction (SPE) columns (comprised of the matrix of C18 reverse phase resin) with 6-8 ml distilled water followed by 6 ml of 70-80% aqueous methanol. The fractions of interest were evaporated *in vacuo* to dryness using a speed-vac. Each fraction was dissolved into distilled water to measure λ_{max}, λ_{min}, and the absorption values at λ_{max} and λ_{min} by spectrophotometry. After drying again by the speed-vac, some of the fractions were subjected to LSIMS, and MS-MS.

Subsequent syntheses of EB-adenine products were carried out following the same procedure to further charaterize the products by spectrophotometry and NMR analysis. After the primary separation and secondary verification of the purity of pooled fractions of interest, the fractions were desalted and dried twice as described above to completely remove salts. In order to measure more precisely the weight of the EB-adenine products of interest, the tubes containing the dry pooled products after speed-vac evaporation were put with phosphorus pentaoxide in a dessicator which was subsequently set *in vacuo* for 3 days before weighing. After desiccation and measuring the weight of the EB-adenine products, they were dissolved in distilled water and a small fraction of each solution was taken to get the absorption values at λ_{max} and λ_{min}, and the corresponding absorptivity coefficients (or extinction coefficients, ε) (pH 7) of each EB-adenine product of interest were determined. Following the same procedure above except for replacing water with HCl (pH 1) or NaOH (pH 13), the absorptivity coefficient of each EB-adenine product, and the associated λ_{max} and λ_{min} at pH 1 or 13 were determined. The unused part of each EB-adenine product in water was desiccated again, and subjected to NMR analysis to characterize its molecular structure.

^{1}H-NMR Spectra Data

^{1}H-NMR spectra of both EB-adenine products was obtained on a Bruker AMX-500 spectrometer in DMSO-d6. EB-Ade I: δ 4.10 (br dd, 1H, J = 13.41 Hz, J = 8.32 Hz, C$\underline{H}_2$–NH), 4.41 (br dd, 1H, J = 13.41 Hz, J = 3.69 Hz, C$\underline{H}_2$–NH), 4.51 (br s, 1H, C$\underline{H}$–OH), 5.13 (d, 1H, J = 10.40 Hz, cis C$\underline{H}_2$=CH), 5.25 (d, 1H, J = 17.20 Hz, tran C$\underline{H}_2$=CH), 5.48 (br s, 1H, CH–O$\underline{H}$), 5.92 (m, 1H, CH_2=C$\underline{H}$), 7.70-8.00 (m, 3H, H–8, N^6–H, and N^7–H), 8.21 and 8.24 p.p.m. (two s, 1H, H-2). EB-Ade II: δ 3.82 (br dd, 1H, J = 13.41 Hz, J = 3.69 Hz, C$\underline{H}_2$–OH), 4.16 (br dd, 1H, J = 13.41 Hz, J = 8.32 Hz, C$\underline{H}_2$–OH), 5.20-5.30 (m, 4H, C$\underline{H}$–CH=C$\underline{H}_2$, and CH_2–O$\underline{H}$), 6.37 (m, 1H, CH_2=C$\underline{H}$), 7.75-8.10 (m, 3H, H–8, N^6–H, and N^7–H), 8.35 and 8.39 p.p.m. (two s, 1H, H-2).

Reaction of EB with Calf Thymus DNA

The procedure described below was also modified from that of Citti *et al.* [24]. Calf thymus DNA (36 mg) was dissolved in 10 mM Tris-HCl buffer pH 7.2 (7 ml) by gently mixing and then EB (334 mg, 4.76 mmol) and methanol (7 ml) were added to the solution. This reaction mixture was heated at 37°C for 18 hours. The reaction mixture was extracted twice with 28 ml diethyl ether to remove unreacted EB. Either neutral thermal hydrolysis or acid hydrolysis was done with samples from the remaining EB-DNA aqueous solution. In the neutral thermal hydrolysis, sample solution in 10 mM cacodylic acid was hydrolyzed by heating at 95°C for 30 min, while in acid hydrolysis, sample solution in 0.1 N HCl was heated at 70°C for 30 min. After neutral thermal hydrolysis, the resulting hydrolysate of each sample went through microconcentration. The neutral thermal hydrolysate (NTH) was chilled to 4°C and 2.5 ml of each sample was pipetted into a Centricon 30 microconcentrator (Amicon, Danver, MA). About 2 ml of hydrolysate was filtered through the

microconcentrator by centrifugation for 1 h at 3000x *g* using a Sorvall Instruments (Dupont Co., Wilmington, DE) RC5C centrifuge. The DNA backbone remained on the Centricon 30 ultrifiltration membrane. The resulting filtrate was subjected to HPLC analysis with the same conditions of eluent, FR and gradient as described in the separation of synthesized EB-guanine adducts. Adduct concentrations were determined by measuring ultraviolet (UV) absorbance (λ=280 nm for all four adducts of interest) and comparing peak areas to calibration curves constructed from the reference adducts. The calibration curves were linear over the range form 50 pmol to 10 nmol of reference adducts.

Guanine Assay and Adenine Analysis

Acid hydrolysates of DNA samples were analyzed for guanine and adenine content using a Whatman (Hillsboro, OR) Partisil 10 SCX column which was eluted with 100 mM ammonium formate, pH 2.8, with 10% methanol, at a flow rate of 1.8 ml/min. Guanine and adenine concentrations of acid hydrolysate were determined by measuring UV absorbance at 254 nm and comparing peak areas to a calibration curve for each.

RESULTS

Reaction of EB with Guanosine

Reaction of EB with guanosine resulted in four main products after acid hydrolysis (Figure 1). The absorption spectra of peak I and II from DAD (Figure 2) resembled well those of EB-Gua I and EB-Gua II shown by Citti *et al.* [24]. The fractions containing each peak were collected during multiple HPLC runs, and pooled when its purity was verified by a spectra match value greater than 995. The HPLC retention time and the spectra of a small portion of the pooled fractions were found to be the same as those of each previous unpooled fraction. After desalting and drying, each product was redissolved into distilled water (pH 7), the λ_{max} and λ_{min} were measured to be 284 nm and 261 nm for both , in agreement with Citti *et al.* [24]. Furthermore, LSIMS of the product identified as EB-Gua II showed a peak of protonated EB-guanine at m/z = 222, and MS-MS of the parent ion at m/z = 222 gave two fragment ion peaks of m/z = 152 and 192 (Figure 3), corresponding well with the fragmentation pattern of EB-Gua II proposed by Citti *et al.* [24].

Reaction of EB with Adenine

Two major products were present in a chromatogram of the primary separation of the reaction mixture of EB with adenine (Figure 4). The absorption spectra of the two major peaks (at retention times of 12.0 and 13.7 min, respectively) from DAD resembled each other with λ_{max} and λ_{min} at 275 nm and 245 nm, respectively (Figure 5). In addition, the λ_{max} and λ_{min} in absorption spectra of the two products also differed from those of any other product. Each fraction corresponding to one of the two peaks of interest was collected during

secondary HPLC separations and then pooled when its purity was verified by a spectra match value (from DAD) greater than 995. After the secondary HPLC analysis, the spectra of a small portion of the pooled fractions were the same as those of previous unpooled fractions from the primary separation, although differences in both flow rate and C18 column changed the retention times of products I and II to 5.9 and 7.4 min, respectively. After desalting and drying, each product was dissolved in distilled water, and the λ_{max} and λ_{min} measured by spectrophotometry were 275 nm and 244 nm for both EB-adenine products. After desiccation, the measured weight of each product and the corresponding absorption value at the measured λ_{max} and λ_{min} either of pH 1 , 7 or 13 for each EB-adenine product were used to calculate the absorptivity coefficients (Table 1).

The LSIMS and MS-MS results for EB-adenine II (at retention time 7.4 min) are shown on Figure 6. LSIMS had a peak of protonated EB-adenine at m/z = 206, and MS-MS of the parent ion at m/z = 206 showed a fragment ion peak of m/z = 136, which is corresponded to $[adenine+H]^+$. The above results suggested that the product was an EB alkylated adenine derivative, but its structure remained ambiguous for the site of EB adduction. In order to delineate definitive structures of both EB-adenine products, ^{1}H-NMR (Figure 7) spectroscopic analysis was conducted. The signals between 7.85 and 8.35 p.p.m. integrated to four protons were assigned to C-8, N-6, N-7, and C-2 protons. Furthermore, the signal for C-6 amino protons integratable to two protons around 6.10 p.p.m. was not found, confirming that both products were N^6-substituted adenine adducts of EB.

Further analysis of NMR spectra showed a downfield chemical shift (5.20-5.30 p.p.m.) for methine proton (C<u>H</u>-N^6) in EB-Ade II when compared to that (C<u>H</u>-OH) in EB-Ade I (4.51 p.p.m.). This clearly indicates that the methinyl carbon in EB-Ade II is adjacent to vinyl and N^6 which cause downfield shift for

the methine proton due to deshielding by both functions and thereby confirms the isomeric regiochemistry of the two EB-adenine products. As the methylene protons in both products are situated adjacent to an optically active carbon (CH), they appear in the NMR as two different signals at 4.10 and 4.41 p.p.m. in EB-Ade I and 3.82 and 4.16 p.p.m. in EB-Ade II. Both the methylene protons appeared as doublet of doublet with equal germinal coupling constant of 13.41 Hz but the the vicinal coupling constants were 8.32 and 3.69 Hz for EB-Ade I and 3.69 and 8.32 Hz for EB-Ade II. Thus, the substitution pattern on the side chain in the products was confirmed. All the evidence from NMR clearly indicated that EB-Ade I and II are N^6-(2-hydroxy-3-buten-1-yl)adenine and N^6-(1-hydroxy-3-buten-2-yl)adenine, respectively, whose structures are shown in Figure 7.

Reaction of EB with Calf Thymus DNA

HPLC analysis with UV detection of the purines released from neutral thermal hydrolysates (NTH) of EB-treated calf thymus DNA showed the presence of 4 DNA adducts in a typical chromatogram (Figure 8). The last two peaks in the NTH solution corresponded to the reference EB-Gua I and II, respectively, with the same retention time. The retention time of the two peaks preceding the last two also matched well with those of the EB-adenine product fractions eluting at 5.8 and 7.2 min, respectively. Furthermore, the addition of a portion of each reference EB-adenine product fraction into a NTH solution sample only augmented the peak area of the corresponding peak without disrupting the pattern of the chromatogram (data not shown). This suggested that the two components eluting prior to the last two in the NTH solution were the two EB-adenine product fractions. Further supportive evidence came from absorption spectra from DAD showing that the spectra of two components

eluting before the last two peaks resembled those of both EB-adenine product fractions, and that the spectra of the last two components were the same as those of two reference EB-guanine adducts. Figure 9 also showed the presence of 4 peaks during HPLC analysis with UV detection of the purines released following acid hydrolysis. The last two corresponded to the reference EB-Gua I and II, respectively, with the same retention time as in standard injections either of the reference EB-Gua I or II alone. The retention time of the second adduct peak matched well with EB-Ade II eluating at 7.2 min. While the first peak may contain EB-Ade I, its identity was confounded by additional UV absorbing material.

The above purified EB-adenine and EB-guanine product fractions were used as reference compounds for the quantification of the relevant EB-DNA base adducts in NTH. Because both the absorptivity coefficients of EB-guanine and EB-adenine adducts I and II were known, the amount of EB-guanine adduct I or II formed in the reaction of EB with calf thymus DNA could be estimated by calibration curves. The estimated amount of purines (including both guanine and adenine) and the resulting proportion of EB-guanine adducts to guanine and the EB-adenine adducts to adenine were determined and listed in Table 2. The amounts of EB-guanine adducts formed in DNA were comparable ($3.9 \times 10^{-2} \pm 0.3 \times 10^{-2}$ EB-Gua I, and $3.2 \times 10^{-2} \pm 0.2 \times 10^{-2}$ EB-Gua II, per guanine) and about one order of magnitude greater than those of EB-adenine adducts I and II in DNA ($1.1 \times 10^{-3} \pm 0.1 \times 10^{-3}$ and $5.2 \times 10^{-3} \pm 2.5 \times 10^{-3}$ per adenine, respectively).

DISCUSSION

The present results from NMR and MS-MS spectra suggest that the structures of two novel EB-adenine products are N^6-(2-hydroxy-3-buten-1-yl) adenine and N^6-(1-hydroxy-3-buten-2-yl) adenine. Two peaks with retention times and UV spectra identical to the two N^6-substituted adenine adducts, as well as two other components identified as N7-substituted EB-guanine adducts, were observed in the neutral thermal hydrolysate of calf thymus DNA exposed to EB. This indicates that these two EB-adenine adducts underwent the thermally facilitative depurination off the DNA backbone and were released into the hydrolysate similar to the N7-substituted EB-guanine adducts [24].

The facilitative release of two N^6-substituted EB-adenine adducts from EB-exposed DNA under the conditions of neutral thermal hydrolysis implies decreased stability of glycosyl bonds of N^6-substituted EB-deoxyadenosine, compared to unmodified deoxyadenosine. This intrinsic instability of glycosyl bonds in N^6-substituted EB-adenine residues may result in apurinic sites, just as N7-substituted EB-guanine residues do. Apurinic sites in mammalian cells have been found to facilitate transition and transversion mutations [27]. Furthermore, interference of base-pairing during DNA replication may arise from a bulky alkyl adduction of EB to adenine on the N^6 position, a critical region for hydrogen bonding between adenine and thymine. Therefore, the presence of the N^6-substituted EB-adenine residues on the parent strand of DNA may cause base-mispairing on the corresponding site of the other DNA strand, and thus induce mutations. This is consistent with the observation of four A:T→G:C tansitions and a A:T→C:G transversion among 18 basepair substitutions in *hprt*-EB mutants [17]. More sensitive methods for the detection and quantitation of

the N^6-substituted EB-adenine residues need to be developed and applied to determine the molecular dosimetry of BD DNA adducts.

Two N^6-substituted deoxyadenosine adducts of EB have also been synthesized from the reaction of EB with 2' deoxyadenosine-3' monophosphate followed by HPLC separation and dephosphorylation, and they were identified in DNA exposed in vivo to EB using a ^{32}P-postlabeling assay [28]. This observation of two EB-deoxyadenosine adducts in EB-exposed DNA is in agreement with the present analysis of neutral thermal hydrolysate from DNA exposed in vitro to EB. Therefore, both methods appear to identify the same EB adduct species of deoxyadenosine either by ^{32}P-postlabeling assay followed by two-dimensional thin layer chromatography (TLC) or by neutral thermal hydrolysis followed by HPLC analysis with UV detection. Although ^{32}P-postlabeling assay with TLC is one of the most sensitive methods for DNA adduct quantitation, the apparent lability of the glycosidic linkage of N^6-substituted deoxyadenosine adducts, as implicated by their presence in neutral thermal hydrolysate of in vitro EB-treated DNA, suggests that it will be important to determine the recovery rate of N^6-substituted deoxyadenosine adducts for ^{32}P-postlabeling studies.

The present result showed that the amount of N^6-substituted adenine adducts formed in calf thymus DNA exposed to EB was approximately one tenth that of EB-guanine adducts. Since nearly equal numbers of point mutations at A:T and G:C base pairs were induced by BD and EB in splenic T-cells of male B6C3F1 mice [17], and the frequency of point mutations at A:T base pairs in the bone marrow of B6C3F1 *lacI* transgenic mice was increased following BD inhalation exposures [25,26], these minor EB-adenine adducts may represent important promutagenic DNA adducts involved in BD-induced mutagenesis and carcinogenesis.

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Table 1. Measured values of $\lambda_{max,}$ and λ_{min} of each EB-adenine adduct , and the corresponding estimated absorptivity coefficients in parenthesis at different pH values.

Types of Adducts	$\lambda_{max}(\varepsilon)/\lambda_{min}(\varepsilon)$ at pH 7	$\lambda_{max}(\varepsilon)/\lambda_{min}(\varepsilon)$ at pH 1	$\lambda_{max}(\varepsilon)/\lambda_{min}(\varepsilon)$ at pH 13
EB-Ade I	275 (12200) /244 (2860)	275.5 (15100) /238 (2940)	274.5 (10900) /238 (2710)
EB-Ade II	275 (12500) /243 (3060)	275 (13600) /238 (2500)	274.5 (10000) /245.5 (2580)

Table 2. Estimated amount of purine adducts in NTH from EB-exposed DNA and the proportion of the amount of EB-adducted purine relative to the parent purine.

Type of DNA Purines or Purine Adducts	Estimated Amount (nmol/mg DNA)	proportion of the EB-adducted purine to the parent purine
Guanine	688 ± 1	
EB-Gua I	27 ± 2	$(3.9 \pm 0.3) \times 10^{-2}$
EB-Gua II	22 ± 1	$(3.2 \pm 0.2) \times 10^{-2}$
Adenine	925 ± 73	
EB-Ade I	0.99 ± 0.01	$(1.1 \pm 0.1) \times 10^{-3}$
EB-Ade II	4.7 ± 2.0	$(5.2 \pm 2.5) \times 10^{-3}$

Data are calculated from two replicate experiments. Each value represents mean ± SEM.

FIGURE LEGENDS

Fig. 1. Chromatogram of the products of the reaction of EB with guanosine after acid hydrolysis. Two main EB-guanine products are refered as EB-Gua I and II.

Fig. 2. Absorption spectra of guanine, EB-Gua I and II in mobile phase during the HPLC analysis with DAD on the previous figure.

Fig. 3. Mass spectra of EB-Gua II from (A) liquid secondary ionization mass spectrometry and (B) MS/MS.

Fig. 4. Chromatogram of the products of the reaction of EB with adenine. Two main EB-adenine products are refered as EB-Ade I and II.

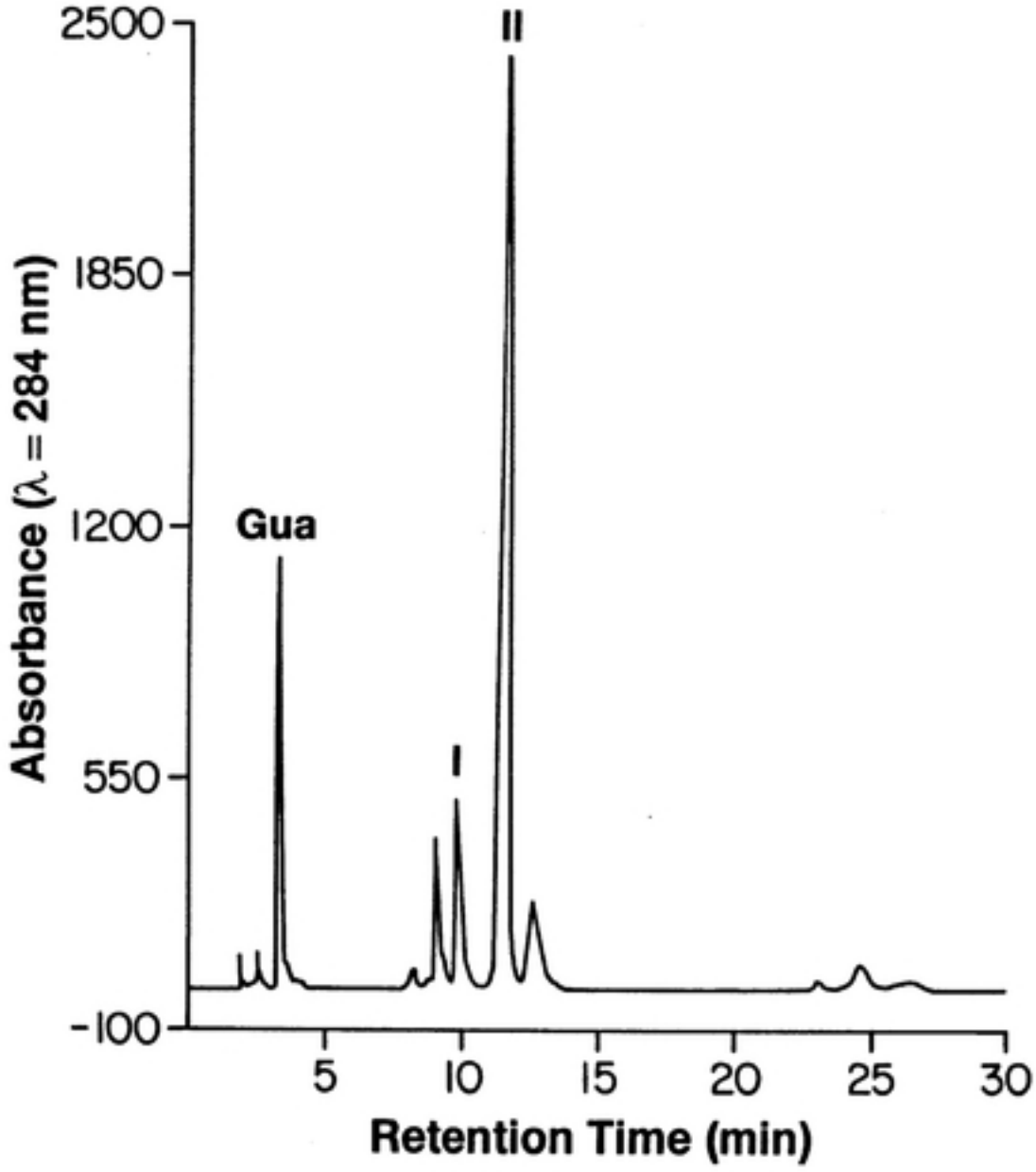
Fig. 5. Absorption spectra of adenine, EB-Ade I and II in mobile phase during the HPLC analysis with DAD on the previous figure.

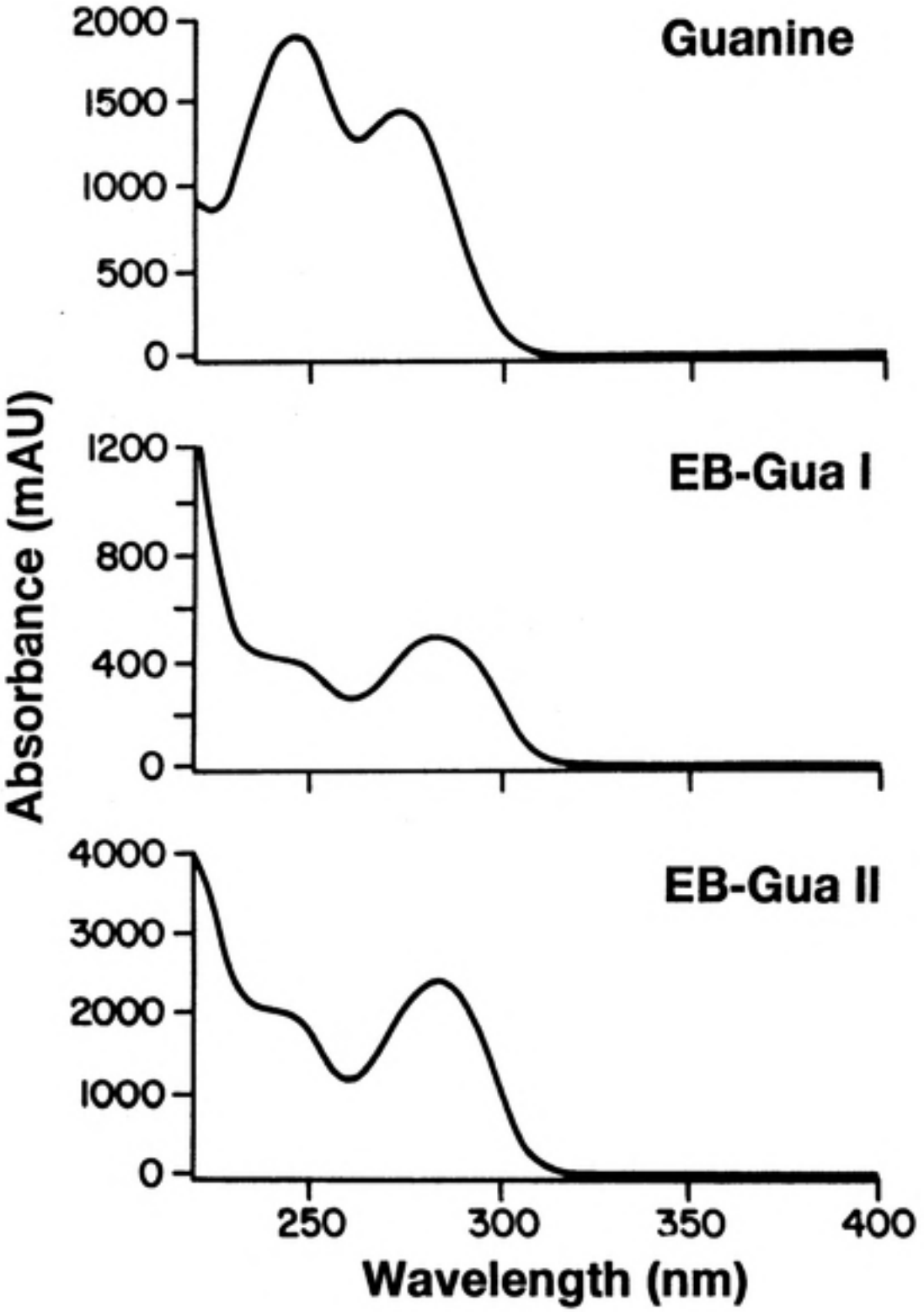
Fig. 6. Mass spectra of EB-Ade II from (A) liquid secondary ionization mass spectrometry and (B) MS/MS.

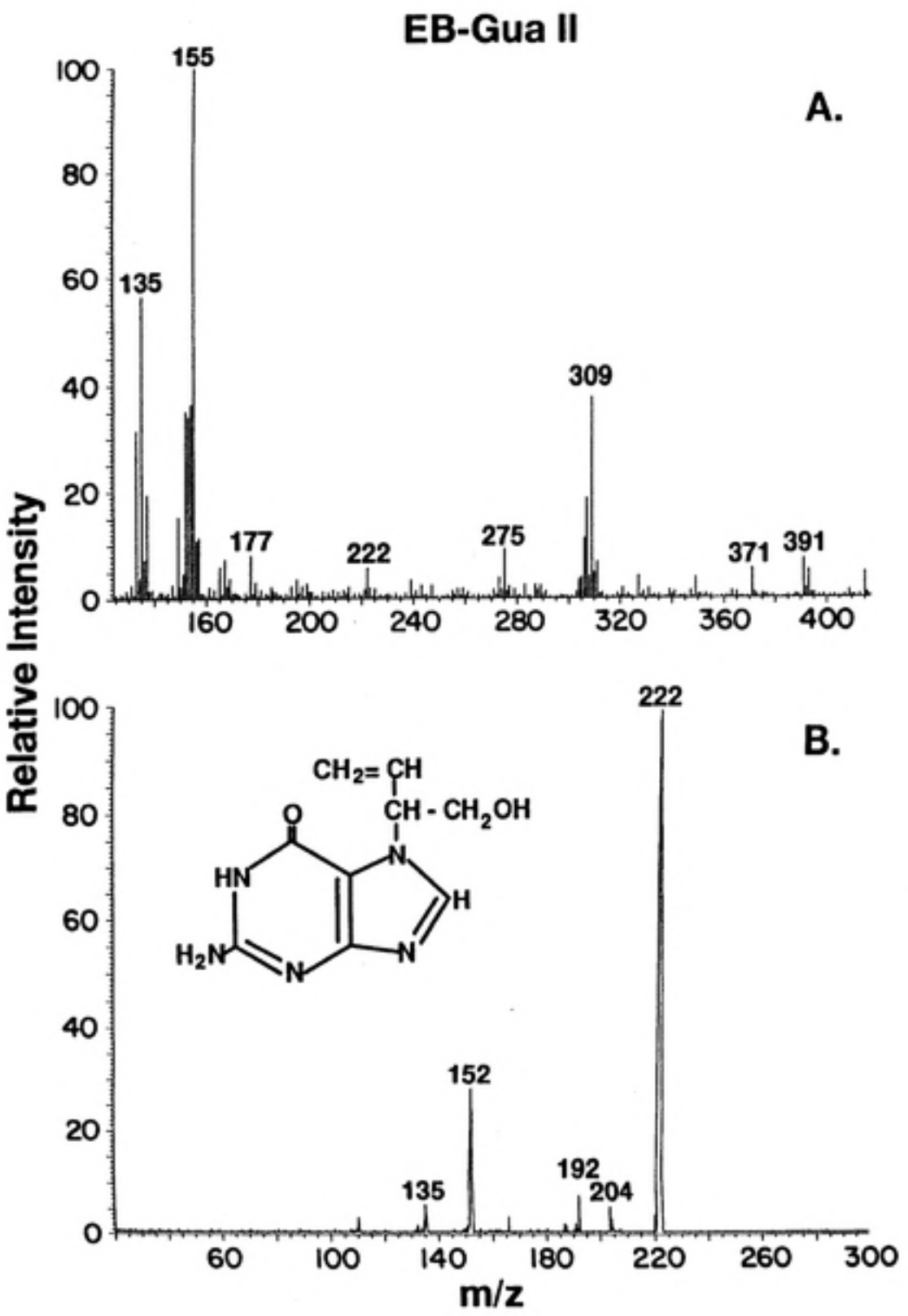
Fig. 7. ^{1}H-NMR spectra of EB-Ade (A) I and (B) II in DMSO-d_6.

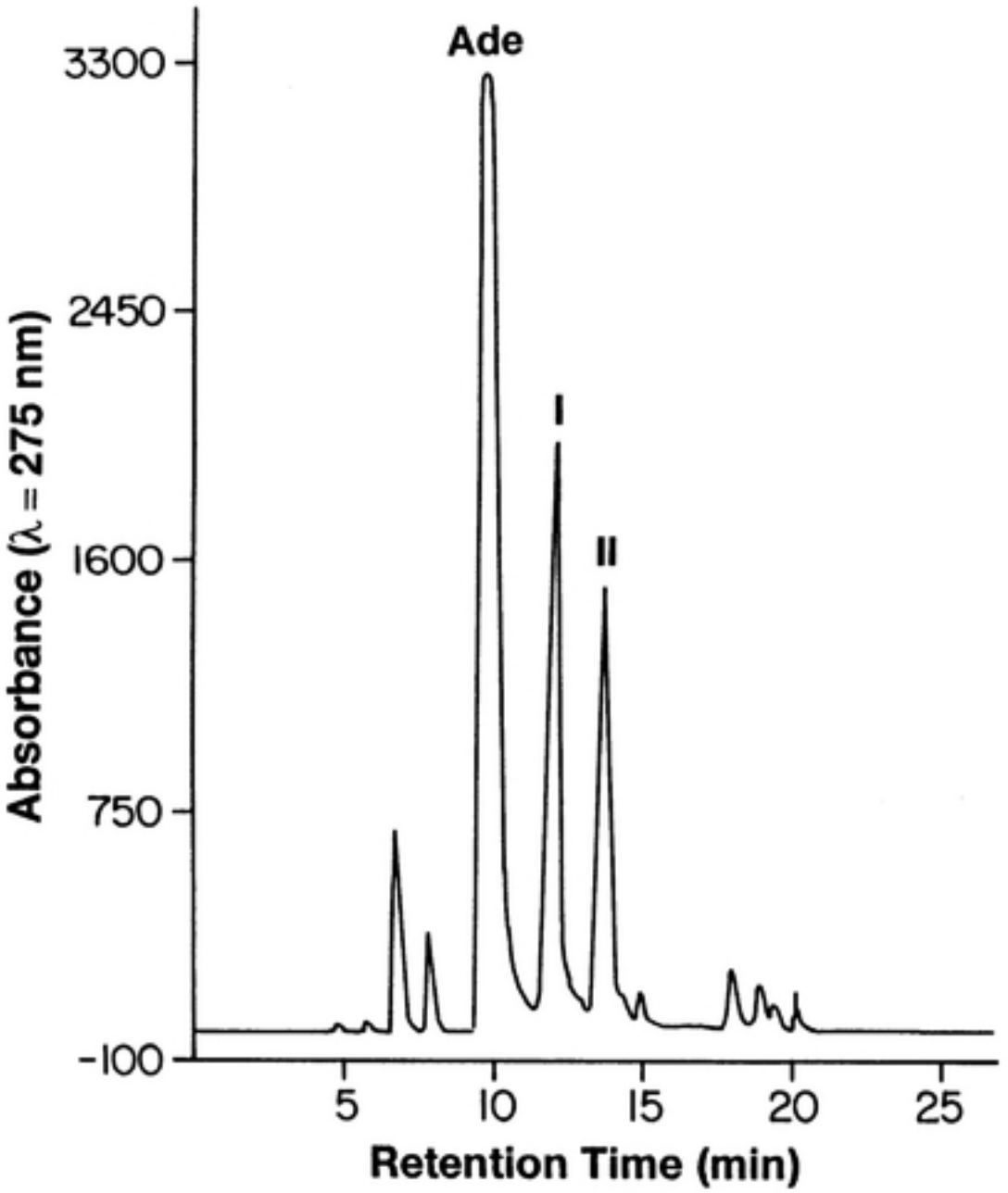
Fig. 8. Chromatogram of neutral thermal hydrolysate from calf thymus DNA exposed to EB.

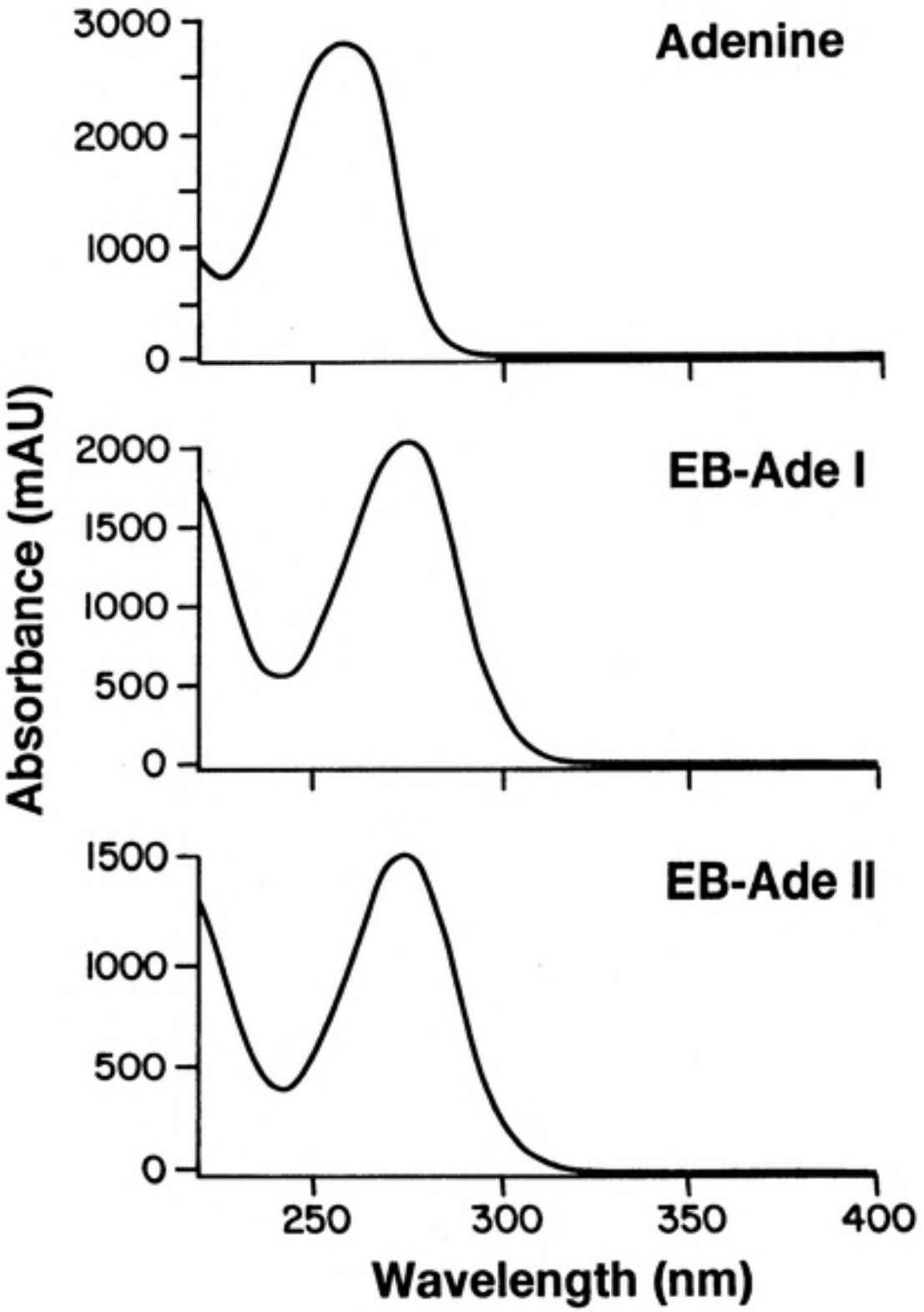
Fig. 9. Chromatogram of acid hydrolysate from calf thymus DNA exposed to EB.

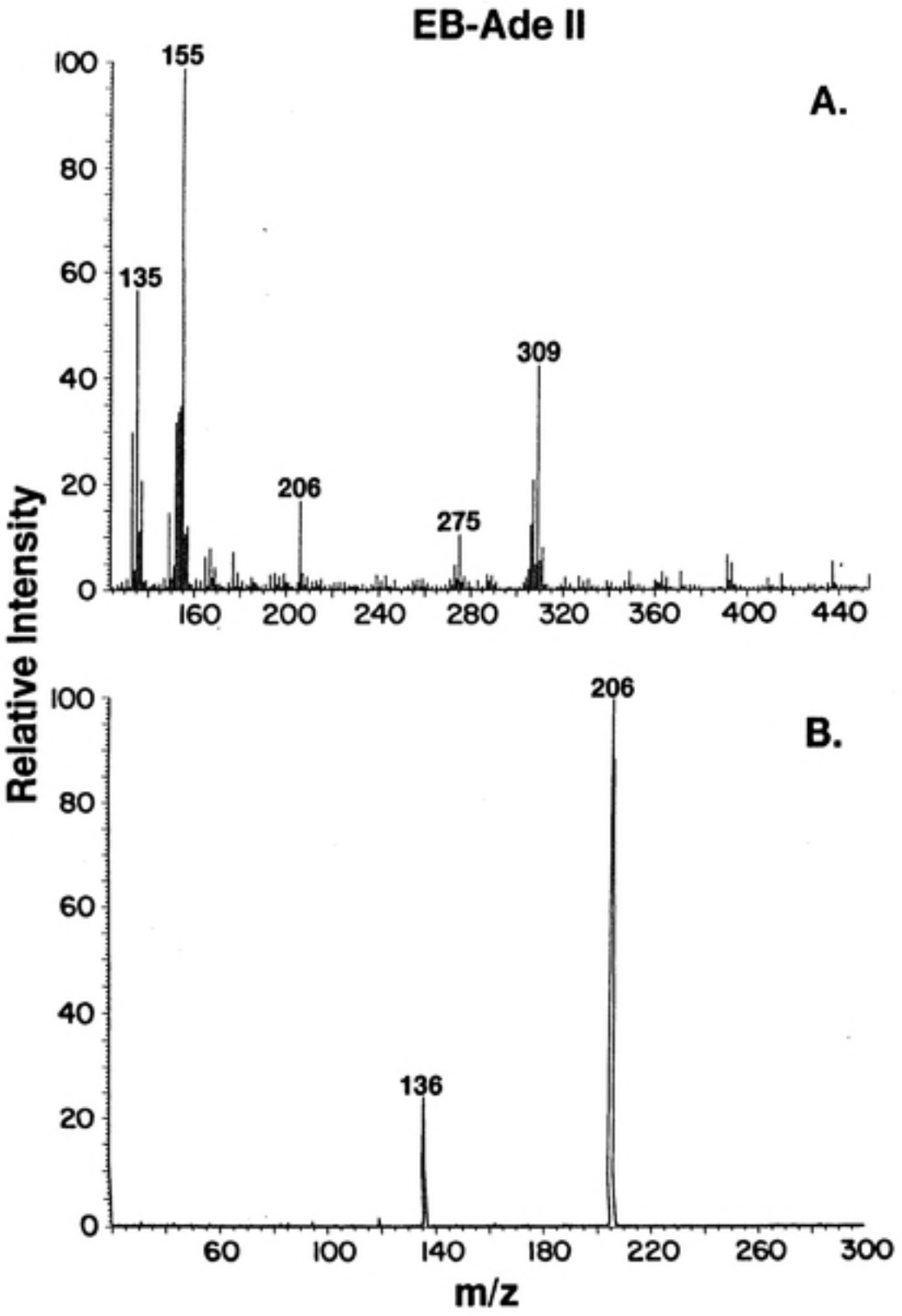

2500
1850
1200
550
-100
Absorbance (λ = 284 nm)
Gua
I
II
5
10
15
20
25
30
Retention Time (min)


Guanine
EB-Gua I
EB-Gua II
Absorbance (mAU)
2000
1500
1000
500
0
1200
800
400
0
4000
3000
2000
1000
0
250
300
350
400
Wavelength (nm)

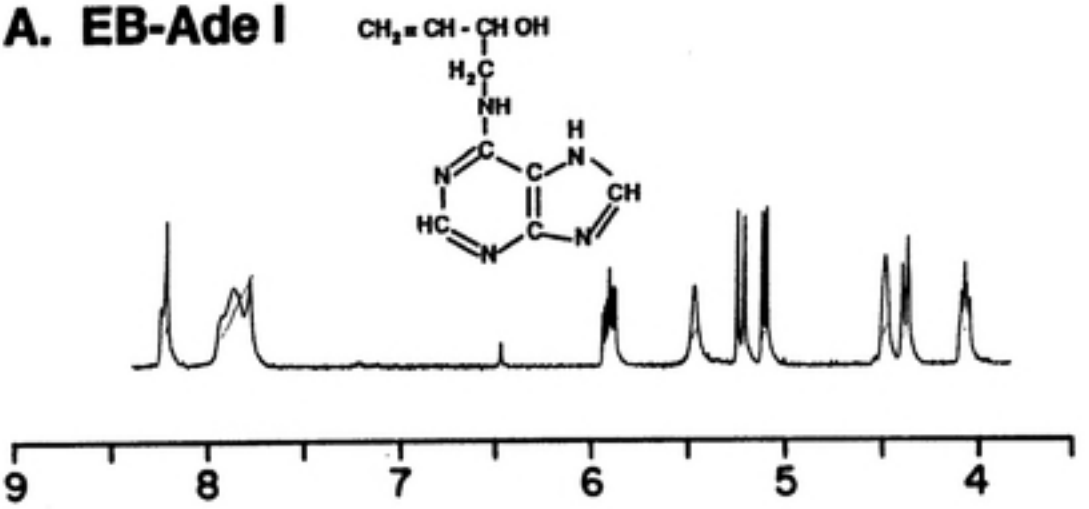

EB-Gua II
A.
B.
Relative Intensity
m/z
155
135
177
222
275
309
371
391
152
192
204
CH2= CH
CH - CH2OH
O
HN
N
H
H2N
N
N


Ade
I
II
3300
2450
1600
750
-100
Absorbance (λ = 275 nm)
5
10
15
20
25
Retention Time (min)

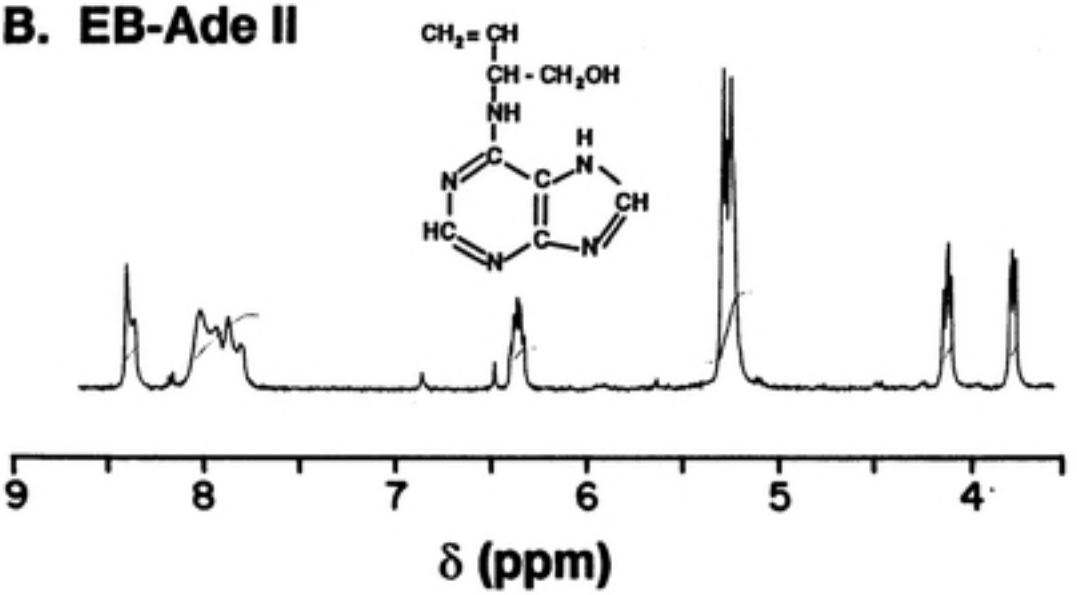

Adenine
EB-Ade I
EB-Ade II
Absorbance (mAU)
3000
2000
1000
0
2000
1500
1000
500
0
1500
1000
500
0
250
300
350
400
Wavelength (nm)

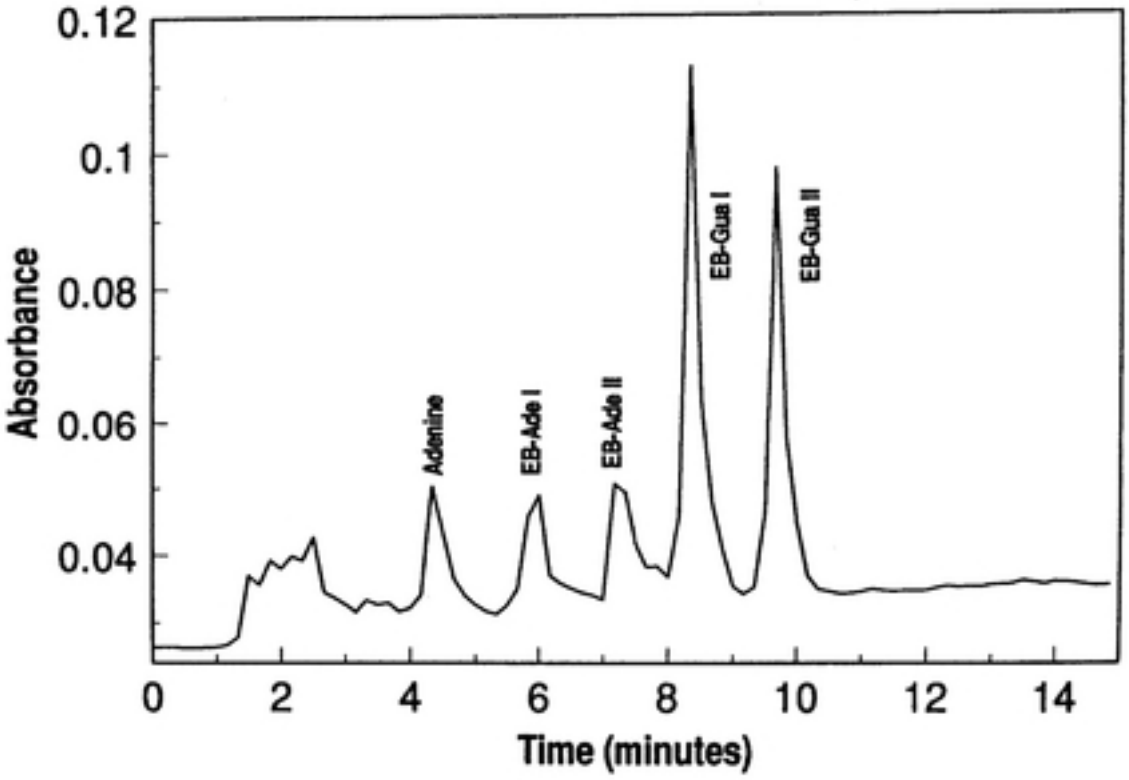

EB-Ade II
A.
B.
Relative Intensity
m/z
155
135
206
275
309
136
206
100
80
60
40
20
0
160
200
240
280
320
360
400
440
60
100
140
180
220
260
300

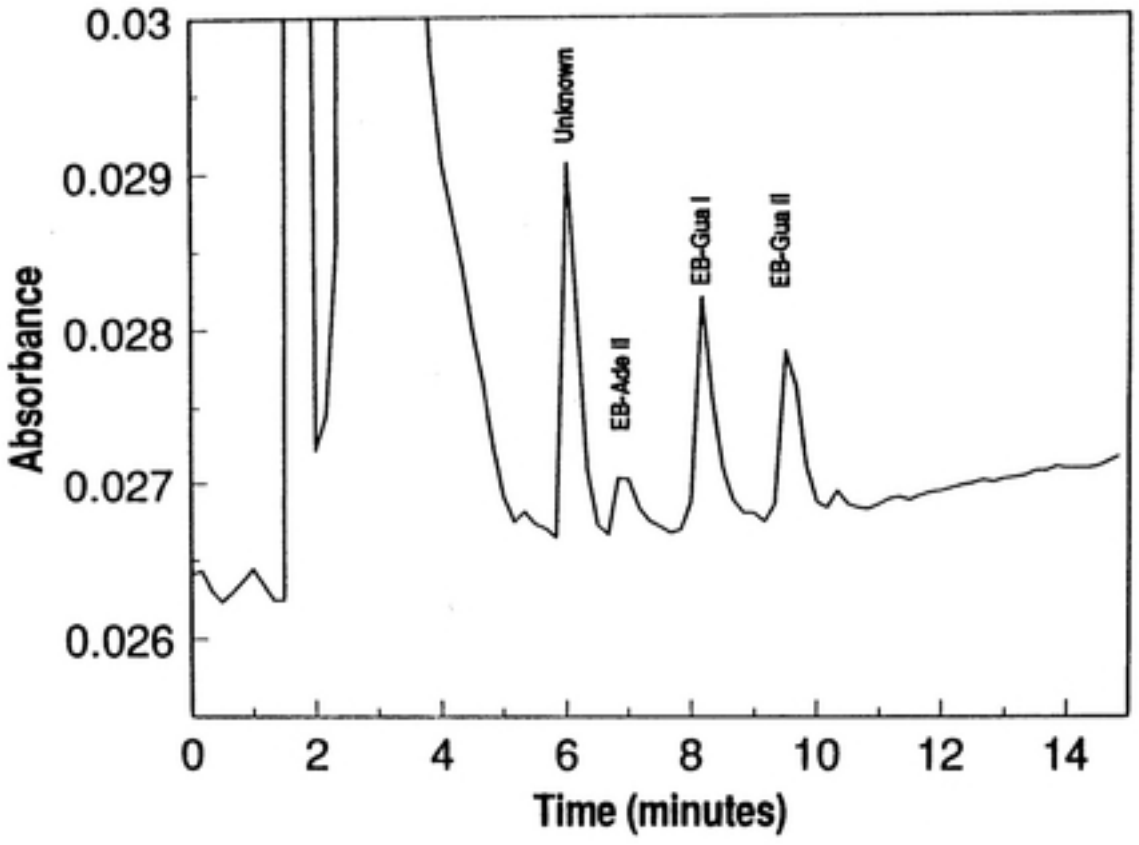
A. EB-Ade I



B. EB-Ade II




0.12
0.1
0.08
0.06
0.04
Absorbance
Adenine
EB-Ade I
EB-Ade II
EB-Gua I
EB-Gua II
0
2
4
6
8
10
12
14
Time (minutes)


0.03
0.029
0.028
0.027
0.026
Absorbance
Unknown
EB-Ade II
EB-Gua I
EB-Gua II
0
2
4
6
8
10
12
14
Time (minutes)